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Full Length Research Paper

Changes of some phenolic compounds and enzyme activities on infected pearl millet caused by *Sclerospora graminicola*

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Downy mildew or green ear disease of pearl millet caused by *Sclerospora graminicola* is the most destructive disease. Affected plants produce green ear with various types of proliferations and malformation of the panicle. Deranged physiology of susceptible and resistant varieties is governed by genetic base, pathogen virulence and induced resistance. Phenolic compounds have been noticed most influential secondary products in determination of resistance in pearl millet plants. In relation to this, activities of polyphenol oxidase (PPO), peroxidase (POX), catalase (CAT) and IAA oxidase (IAAO) have also been found deranged considerably in the downy mildew affected plants of susceptible and resistant cultivars. The study suggests that accumulation of total phenols and OD-phenols caused the hyperphenolicity in infected resistant host tissues despite increased activities of POX and PPO. Total amino acids and free proline contents were increased manifold (1222.2 and 942.6%, respectively) in diseased tissues, particularly in resistant cv. HHB 67 than in susceptible one (Eknath), indicating biotic stress caused by *S. graminicola*. The role of enzyme activities and their related compounds have been discussed in the present paper.

Key words: Pearl millet, downy mildew, *Sclerospora graminicola*, metabolites, oxidative enzymes, hyperphenolicity.

INTRODUCTION

Pearl millet {*Pennisetum glaucum* (L.) R.Br.} is the staple food crop of a large section of peasant community in tropical regions and is the major component of sustainable farming systems in the arid and semi-arid regions. Downy mildew (DM) disease caused by *Sclerospora graminicola* (Sacc.) Schroet is the most serious constraint in realizing higher production of pearl millet causing up to 80% loss in grain yield.

Phenolic compounds are among the most influential and widely distributed secondary products in the plants. Such compounds govern disease resistance in many crop plants. Increased activity of polyphenol oxidase (PPO), peroxidase (POX), and phenylalanine ammonia-lyase (PAL) has been reported in plants treated with various biotic and abiotic inducers of resistance (Huang and Backhouse, 2005; Raghvendra et al., 2007). *Sclerospora graminicola* is responsible for causing downy mildew disease in pearl millet cultivars. In the present study, biochemical changes in susceptible and resistant cultivars of pearl millet (*Pennisetum glaucum* (L.) R. Br.) were estimated on the basis of enzyme activities of peroxidase (POX), polyphenol oxidase (PPO), catalase (CAT) and IAA oxidase (IAAO).

Susceptible and resistant pearl millet cultivars infected with downy mildew (DM), were used for understanding biochemical mechanism of disease resistance.

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MATERIALS AND METHODS

Host and pathogen

Based on the study conducted by Rao et al. (2005) pearl millet cultivars susceptible (cv. Eknath 301) and resistant (HHB 67) to downy mildew were selected for the experiment. Seeds of pearl

Table 1. Changes in phenols and amino acids in healthy (H) and diseased (D) tissues of resistant (R) and susceptible (S) genotypes of pearl millet.

Genotypes	Type of material	Total phenols	OD phenols	Total free amino acids	Free proline
		(mg g^{-1} dry wt)			(mg $^{-1}$dry wt)
Eknath (S)	Ear head (H)	1.78	1.53	2.52	0.37
	Ear head (D)	3.45	0.71	10.42	2.10
		(+93.4)*	(- 53.5)	(+ 312.0)	(+ 454.9)
HHB-67 (R)	Ear head (H)	1.62	1.59	1.22	0.14
	Ear head (D)	7.01	1.34	16.08	1.50
		(+312.7)	(- 15.8)	(+ 1222.2)	(+ 942.6)
CD 5%		0.43	0.06	0.35	37.74

*Figures in the parenthesis are % changes in diseased tissues over healthy.

millet cultivars Eknath 301 and HHB 67 obtained from the millet breeder of Central Zone Research Institute (CAZRI), Jodhpur, India, were used throughout the study. Plants were raised in downy mildew sick-plot maintained since 1995 in Central Research Farm of CAZRI, Jodhpur, containing heavy load of soil borne oospores of highly virulent Jodhpur pathotype of *S. graminicola* (Thakur et al., 1998). Additionally sporangial inoculum was provided by the infector-row system as described by Williams et al. (1981). Disease free (control) plants of both the cultivars were raised from the seeds pre-treated with systemic fungicide metalaxyl formulation Apron 35SD at 6 g kg^{-1} concentration. The crop was fertilized with diammonium phosphate (40 kg ha^{-1}) as basal dose before sowing and irrigated at 2 week intervals. No insecticides or herbicides were applied.

Metabolites and enzymes estimations in pearl millet cultivars

The green leaves were separated and cut into small uniform pieces. From this, representative samples of 500 mg were taken from 50 day old plants of each cultivar for the estimation of total and ortho-dihydroxy (OD) phenols. Total phenols and OD phenols were analyzed by adopting methods given by Bray and Thorpe (1954) and Mahadevan and Sridhar (1986). Free proline was determined using the method suggested by Bates et al. (1973). Proteins were estimated by the method of Lowry et al. (1951).

In order to ascertain the role of some antioxidant enzymes, which are important markers for resistance, in the cultivars known for their resistance (HHB 67) and susceptibility (Eknath) the activity of defense related enzymes was observed in both the cultivars. The enzymes peroxidase (POX) and polyphenol oxidase (PPO) were estimated using the method suggested by Shannon et al. (1966) and Kar and Mishra (1976). The assay mixture of POX contained 2.3 mL of 0.1mL of phosphate buffer (pH 6.0) at 4°C. The reaction mixture (0.5 mL) consisted of 0.01 M pyrogallol and 0.1 mL of 0.025 M hydrogen peroxide. The addition of 0.1 mL of crude enzyme extract initiated the reaction, which was measured spectrophotometrically at 420 nm (Systronics spectrophotometer, Ahmedabad, India). The assay mixture of Polyphenol oxidase (PPO) contained 1.5 mL of 0.1 M phosphate buffer (pH 6.0) at 4°C. The reaction mixture (0.5 mL) consisted of 0.01 M pyrogallol. The addition of 1.0 mL of crude enzyme extract initiated the reaction, which was measured spectrophotometrically at 420 nm at 30 s interval for 3 min. Enzymes catalase and IAA oxidase were estimated as reported by Mahadevan and Sridhar (1986). For catalase (CAT) the reaction mixture contained 2.7 mL of 0.1 M phosphate buffer (pH 6.5) at 4°C. The reaction mixture (0.1 mL) consisted of 0.2 M hydrogen peroxide. The addition of 0.2 mL of crude enzyme extract initiated the reaction, which was measured spectrophotometrically at 230 nm at 15 s interval for 2 min. For IAA oxidase (IAAO) the reaction mixture contained 3.0 mL of 0.1M phosphate buffer (pH 6.0) at 4°C. The reaction mixture (2.25 mL) consisted of 2, 4- dinitrophenol (1mM) and 0.25 mL manganese chloride (0.5 mM). The addition of 1.0 mL of crude enzyme extract and 0.1 mM IAA solution (1.5 mL) with ferric chloride (anhydrous) (0.5 mM) initiated the reaction, which was measured spectrophotometrically at 530 nm. All the estimations were done in triplicate and the results on fresh weight basis are statistically analyzed and reported.

RESULTS

Proline

Free proline contents increased manifold (942.6%) in diseased ear head of resistant HHB 67 and susceptible Eknath cultivars (454.9%) in comparison to healthy ones (Table 1). However, free proline content was higher in healthy susceptible cultivar Eknath than resistant variety HHB 67. Results indicated that free proline increased tremendously on infection of green ear disease in resistant variety when compared to normal ear heads. These results are substantiated with increase in total amino acid contents in diseased tissues of susceptible as well as resistant pearl millet cultivars. In total amino acid content highest accumulation was recorded in HHB-67 cultivar (1222.2%).

Phenols

Increased total phenols were observed in green ear infected tissues of HHB 67 (7.01 mg/g dry wt) in comparison to healthy counterparts (1.62 mg g^{-1} dry wt). Likewise phenols were higher in infected susceptible Eknath cultivar (3.45 mg g^{-1} dry wt) than normal tissues.

Table 2. Changes in enzymes in healthy (H) and diseased (D) tissues of resistant (R) and susceptible (S) genotypes of pearl millet.

Genotypes	Type of material	Polyphenol-oxidase (PPO)	Peroxidase (PO)	IAA oxidase	Catalase	Soluble protein
		(OD min^{-1} mg^{-1} protein)				(mg g^{-1} dry wt.)
Eknath (S)	Ear head (H)	0.0186	2.503	0.0072	0.2073	23.13
	Ear head (D)	0.0155	3.431	0.0205	0.4145	31.75
		(-16.6)*	(+37.1)	(+ 188.9)	(+ 99.9)	(+ 37.3)
HHB-67 (R)	Ear head (H)	0.0110	1.514	0.0083	0.2286	17.46
	Ear head (D)	0.0201	16.235	0.0094	0.1755	37.12
		(+ 82.7)	(+ 972.3)	(+ 13.3)	(-23.2)	(+ 112.5)
CD 5%		0.0012	0.694	0.0065	0.0039	0.63

*Figures in the parenthesis are % changes in diseased tissues over healthy.

However, OD-phenol contents decreased maximum in susceptible than resistant pearl millet plant tissues (Table 1).

Enzymes

Results revealed that activity of polyphenol oxidase (PPO) was maximum (82.7%) in completely malformed ear-heads of HHB 67 whereas in susceptible Eknath cultivar decreased (16.6% from healthy counter parts). In case of diseased ear-heads increased PPO activity was recorded only in resistant cultivar. Peroxidase (POX) activity was also greatly increased in green ear affected ear heads of resistant pearl millet cultivar (increased 972.3% over healthy) in comparison to the healthy as well as susceptible ones (Table 2). However, the maximum IAAO activity was observed in susceptible Eknath cultivar (Table 2). Interestingly, the catalase activity was higher in susceptible (99.9%) than resistant cultivar. Similarly, soluble proteins were higher in diseased ear heads of resistant HHB 67 that is, 112.5% than Ekanth susceptible variety (37.3%).

DISCUSSION

Downy mildew of pearl millet is a typical case of inflorescence malformation and conversion of florets into green leafy structures. Earlier investigations suggested that in abnormal growth of ear heads, PPO activity always remained higher in comparison to completely proliferated suppressed and normal ear heads (Shekhawat et al., 1984). Phenols and oxidizing enzymes such as PPO and POX have an active role in resistance mechanism of plant diseases. It has been reported that resistant cultivars have higher amount of total and OD-phenols (auxin protectors) than susceptible ones (Lily and Ramadasan, 1979; Sharma et al., 1983; Luthra et al., 1988). In present study higher-level of total phenols (hyperphenolicity) were recorded in resistant cultivar HHB 67 than susceptible. Further, results indicated that resistant pearl millet variety contained higher amount of OD phenols. Gupta (2001) has reported higher phenolic contents in leaves and roots of resistant pearl millet than susceptible varieties. In case of crown gall tissues of sunflower (Stonier, 1972) and in *Zizyphus jujuba* stem galls incited by a mite *Eriophyes cernuus* accumulation of phenols has active role in hyperauxinity in producing abnormal growths of plants (Tandon, 1976) . The phenomenon of the free-proline accumulation in plants exposed to diverse environmental and biological stresses has considerable physiological significance. In addition to the water stress, salinity also induces accumulation of free proline in plants. Moreover, during pathogenesis in plants by microorganisms proline contents increased in many folds in susceptible and resistant cultivars (Raj et al., 1983; Sinha et al., 1983, Gupta, 2001).

Increase in oxidizing enzymes particularly PPO and POX has tremendous impact on host physiology and predominantly genes responsible for the resistant pearl millet cultivars (Thukral et al., 1986; Shetty et al., 2001). Further, Niranjanraj et al. (2006) have found that seedlings of resistant varieties had greater PPO activity than susceptible seedlings. Inoculated seedlings had significantly higher PPO levels than uninoculated seedlings. In present results also higher PPO activities in ear head tissues infected with DM fungus of resistant pearl millet strongly support the views expressed by Shetty et al. (2001). Similar phenomenon has also been observed in pear fruits infected resistant cultivar with *Erwinia amylovora* pathogen (Honty et al., 2005). Thukral et al. (1986) found that activities of POX and PPO are linearly related to the degree of resistance at both the 30- and 50-day growth stages. The defense-related enzymes and isoenzymes pattern of ß-1; 3-glucanase and POX in the seedlings of different generations indicated that the resistant populations showed higher enzyme activities

(Shetty et al., 2001). But, activity exhibited slightly decreased in completely proliferated ear heads than normal ones (Shekhawat et al., 1984). These phenols formed highly active quinines compounds (Webb, 1966). Noticeably, the low PPO activity in diseased ear heads of susceptible cultivar may be due to accumulation of phenols. Polyphenol oxidase is reported to act on stimulatory towards the IAA oxidase activity at low concentrations and inhibitory at higher concentrations (Kosuge, 1969). In the present case, the PPO activity was higher but as the OD phenols increased PPO became inactive. Once the oxidation of these phenols was inhibited, this enzyme increased considerably resulting in IAA oxidase inhibition (Shekhawat and Arya, 1979). Thus, it can be inferred that the accumulation of OD-phenols inhibited IAA oxidation on the malformed ear heads.

As far as activity of catalase is concerned, Rudolph and Stakmann (1964) have suggested impact of catalase on the virulence of pathogen on the host-parasite co-existence. The role of catalase as inhibitor of IAA oxidase has also been emphasized by Lane and King (1968). In the present work, varied level of CAT activity was observed in both resistant and susceptible cultivars. Resistant cv. HHB 67 showed a significant drop in CAT activity. In case of cowpea against root rot caused by *Rhizoctonia* solani, resistant cultivar exhibited maximum decline in CAT activity (Chandra et al., 2001). Peroxidase is one of the important pathogenesis -related proteins (PR-Proteins) . It has dual role in plant defense mechanisms, one as its involvement in reactive oxygen intermediates (ROI) metabolism to generate hydrogen peroxide, and secondly, it is capable of reducing the level of hydrogen peroxide during H_2O_2- dependent polymerization of hydroxyl cinnamoyl alcohols (lignin bio-synthesis) (Bolwell et al., 1995; Monties, 1989). Therefore, timing and localization of increased POX activity (Tandon, 1976) and affinity for substrates for lignification, as well as for the formation of H_2O_2, clearly suggests that POX is involved in formation of barrier substances confining to the site of pathogen penetration (Gay and Tuzun, 2000; Pomar et al., 2002). In pearl millet cultivars, association of POX activity with resistance to DM has been reported by Manjunatha et al. (2008) observing maximum POX activity in the highly resistant variety. Similarly in the present study, a considerable increase (972.3 times) in POX activity in diseased ear head of resistant cv. HHB 67 is indicative of the role of POX in inducing resistance. in catalase activity can be attributed to the lower IAA oxidation in *S. graminicola* induced green ear heads. Thus, increased PPO activity, phenolics and free-proline are positively correlated with downy mildew resistance in pearl millet cultivars under field conditions. PPO may be actively involved in plant defense mechanism and can be used as a marker of resistance to downy mildew infection in pearl millet.

In recently concluded study on elicitation of resistance and defense related enzymes by amino acids and raw cow's milk in pearl millet against downy mildew disease (Arun Kumar et al. 2009) on inducing resistance in pear millet against downy mildew disease using amino acids and raw cow milk (RCM) increased activities of enzymes (PAL, PO and -1, 3-glucanase) were recorded in both RCM and amino acids treated DM susceptible (cv. 7042S) plants suggesting their active role in inducing defense responses in host suppressing downy mildew disease in pearl millet. Raw cow's milk and amino acids have emerged as non-phytotoxic natural resources, which activate the host defense responses during pathogenesis. In this case activation of induced resistance may be correlated with amino acid-mediated phenylpropanoid pathway. The present study thus amply indicates that DM infection in pearl millet plants increases accumulation of total and OD-phenols along with certain oxidizing enzymes resulting in hyperphenolicity in resistant host tissues.

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